



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : A61K 38/05, 31/40	A1	(11) International Publication Number: WO 98/35691 (43) International Publication Date: 20 August 1998 (20.08.98)
(21) International Application Number: PCT/US98/03077		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).
(22) International Filing Date: 17 February 1998 (17.02.98)		
(30) Priority Data: 08/801,936 15 February 1997 (15.02.97) US 08/988,339 3 December 1997 (03.12.97) US		
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(54) Title: TREATMENT OF INFARCTS THROUGH INHIBITION OF NF-KAPPAB		
(57) Abstract		
<p>This invention is directed to treating ischemia by administering proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF-�B via the ubiquitin proteasome pathway, or mixtures thereof.</p>		

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TREATMENT OF INFARCTS THROUGH INHIBITION OF NF-KAPPAB

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10 FIELD OF THE INVENTION

This invention relates to treatment of ischemia and reperfusion injury, including preventing or reducing the size of infarct after vascular occlusion.

15 BACKGROUND OF THE INVENTION

All tissues are sensitive to hypoperfusion and the resulting lack of oxygen, ischemia. Prolonged ischemia will result in cellular damage. The magnitude of the injury and the potential for tissue rescue depends upon the degree and duration of the ischemia. With long ischemic periods, cellular death occurs (infarction) and under these conditions the injury is irreversible. On the other hand, dying cells or cells targeted for cell death may be rescued by drug treatment, if applied in a timely fashion.

20 Major ischemic events of therapeutic concern include, but are not limited to, heart attacks and stroke. In man, stroke accounts for 10% of all premature deaths, and of those that survive the insult, 50% are left severely disabled. Only a small fraction, 10%, of patients actually recover full function.

25 Over 1,500,000 Americans suffer from myocardial infarctions each year. About half of these do not survive to reach the hospital. However, with the acceptance of thrombolytic therapy such as streptokinase or tissue plasminogen activator (TPA), the one month survival rate for patients who do reach the hospital is as high as 93.6% (Werns, S.W. *Textbook of Interventional Cardiology*, ed. Topol, E.J. WB Saunders: 1994, pp142-153). By lysing the clot early in the course of infarct, ischemic muscle and tissue can be salvaged. However, reperfusion in and of itself leads to tissue damage.

30 Reperfusion injury may occur as a result of one or more of the following events: cellular acidosis leading to calcium overload; increased intracellular osmotic loads of

catabolites leading to cell swelling; free radicals from neutrophils and other inflammatory cells.

Neutrophils are seen in reperfused myocardium shortly after reperfusion. Monocytes/macrophages appear within 24 to 48 hours. Neutrophil infiltration is three to five fold greater in reperfused myocardium than in ischemic myocardium, is initiated by adhesion to endothelial cells, and occurs within 10 minutes of reperfusion. Neutrophils in and of themselves may become trapped in capillaries and impede reperfusion. Intravascular neutrophils may block up to 27% of the capillaries, and have been shown to be related to decreased regional blood flow (Forman et al., *Acute Myocardial Infarction*, eds. Gersh et al. Elsevier: 1991, pp 347-370). This can result in the "no-reflow" phenomenon, where blood flow continues to decrease after reperfusion.

It is known that neutrophils must first adhere themselves to the endothelial cell wall through the interactions with adhesion molecules. Once attached to the vessel cell wall, the neutrophils then force themselves between adjacent endothelial cells and move into the brain tissue, where they release cytotoxic cytokines. The expression of such adhesion molecules is increased following cell damage including ischemia. In addition, endothelial cell walls become more permeable to infiltrating cells due to the release of nitric oxide (NO). Agents that inhibit the movement (diapedesis) of neutrophils from surrounding blood vessels into the damaged tissue may thus be of value in allowing dying cells time to recover from the ischemic insult.

There is a need in the art for effective therapies to prevent or reduce the consequences of ischemia.

SUMMARY OF THE INVENTION

In a first aspect, the present invention is directed to a method of treating ischemia in
5 a mammal comprising administering to said mammal an effective amount of an NF- κ B activation inhibitor. Preferred NF- κ B activation inhibitors are selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, inhibitors of serine phosphorylation of I κ B- α . and mixtures thereof. Preferably, the agent is administered to the mammal after the onset of transient vascular occlusion and prior to induction of
10 permanent ischemic damage.

In a second aspect, the present invention is directed to a method of preventing or lessening the severity reperfusion injury in a mammal comprising administering to said mammal an effective amount of an NF- κ B activation inhibitor. Preferred NF- κ B activation inhibitors are selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, inhibitors of serine phosphorylation of I κ B- α . and mixtures thereof.
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In a third aspect, the present invention is directed to a method of preventing, reducing the size of, or lessening the severity of infarction in a mammal comprising administering to said mammal an effective amount of an NF- κ B activation inhibitor. Preferred NF- κ B activation inhibitors are selected from the group consisting of proteasome
20 inhibitors, ubiquitin pathway inhibitors, inhibitors of serine phosphorylation of I κ B- α . and mixtures thereof. In preferred embodiments, the method according to this aspect of the invention prevents or lessens severity of infarction after occlusion of a cerebral vessel or a cardiac vessel. In certain preferred embodiments, the method prevents the occlusion from resulting in stroke, or lessens the severity of a stroke resulting from cerebral vessel
25 occlusion.

In a fourth aspect, the present invention is directed to a method of treating ischemia or reperfusion injury, including preventing or lessening the severity of infarction in a mammal comprising administering to the mammal an adjunct therapeutic, in addition to administering an NF- κ B activation inhibitor. Preferred NF- κ B activation inhibitors are

selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, inhibitors of serine phosphorylation of I κ B- α . and mixtures thereof. Certain preferred adjunct therapeutics include without limitation, agents such as steroids which further inhibit NF- κ B activation or inhibit the expression or action of proinflammatory cytokines or cellular adhesion molecules; agents which act to either reperfuse or oxygenate tissues, such as antiedema drugs, thrombolytics such as TPA, streptokinase and urokinase, polyanions such as heparin, anticoagulants; and agents that assist in temperature normalization.

5

Preferred NF- κ B activation inhibitors inhibit NF- κ B activation by the ubiquitin-proteasome pathway. In certain preferred embodiments, the NF- κ B activation inhibitor inhibits phosphorylation of I κ B- α . In certain preferred embodiments, the NF- κ B activation inhibitor is a proteasome inhibitor. Preferably, the proteasome inhibitor is selected from the group consisting of peptidyl aldehydes, boronic acids, boronic esters, lactacystin, and lactacystin analogs. In certain preferred embodiments, NF- κ B activation inhibitor is a ubiquitin pathway inhibitor.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the cascade of NF- κ B activation leading to reperfusion injury, 5 including infarct. Points of intervention by the methods according to the invention are indicated.

Figure 2 shows the ubiquitin-proteasome pathway.

Figure 3 is directed to reduction of infarct volume following middle cerebral artery (MCA) occlusion by administration of the proteasome inhibitor *N*-(2-pyrazine)carbonyl-L-phenylalanine-L-leucine boronic acid. 10

Figure 4 is directed to reduction of infarct size, expressed as a percentage of the contralateral hemisphere, by administration of the proteasome inhibitor *N*-(2-pyrazine)carbonyl-L-phenylalanine-L-leucine boronic acid.

Figure 5 is directed to reduction of infarct volume by administration of the 15 proteasome inhibitor 7-n-propyl-clasto-lactacystin β -lactone following middle cerebral artery (MCA) occlusion.

Figure 6 is directed to reduction of neurological score by administration of the proteasome inhibitor 7-n-propyl-clasto-lactacystin β -lactone following middle cerebral artery (MCA) occlusion.

Figure 7 is directed to reduction of infarct volume by administration of the 20 proteasome inhibitor 7-n-propyl-clasto-lactacystin β -lactone.

Figure 8 is directed to reduction of neurological score by administration of the proteasome inhibitor 7-n-propyl-clasto-lactacystin β -lactone.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to treatment of ischemia and reperfusion injury, including preventing, reducing the size or lessening the severity of infarct after vascular occlusion.

5 The patent applications, patents and literature references cited herein indicate the knowledge in this field and are hereby incorporated by reference in their entirety. In the case of inconsistencies the present disclosure will prevail.

10 It has now been unexpectedly discovered that the ubiquitin-proteasome pathway is a target for treating ischemia and reperfusion injury, including preventing, reducing the size, or lessening the severity of infarcts following vascular occlusions such as occur during heart attack or stroke, and that inhibitors of NF- κ B activation via the ubiquitin-proteasome pathway can provide effective therapy for these conditions. The invention provides surprisingly effective methods for treating ischemia or reperfusion injury.

15 The present inventors have discovered that blocking proteasome function reduces the effects of ischemia, such as reducing infarct size following vascular occlusion. This can be done by direct proteasome inhibition (shown with *N*-(2-pyrazine)carbonyl-L-phenylalanine-L-leucine boronic acid and 7-n-propyl-clasto-lactacystin β -lactone) or by blocking ubiquitination of proteasome-targeted proteins such as 20 I κ B- α . Any inhibitors which affect activation of NF- κ B via the ubiquitin/proteasome pathway in eukaryotic cells are expected to be effective in preventing or treating infarction, including infarction following vascular occlusion and are thus in the scope of the present invention.

25 In accordance with the present invention, treatment of ischemia, including reperfusion injury, prevention of infarction and reduction in size or lessening of severity of infarct is achieved by administering to a mammal an effective amount of an NF- κ B activation inhibitor. Preferred NF- κ B activation inhibitors are selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, inhibitors of serine phosphorylation of I κ B- α . and mixtures thereof.

30 In a first aspect, the present invention is directed to a method of treating ischemia in a mammal comprising administering to said mammal an effective amount of an NF- κ B activation inhibitor. All tissues are sensitive to lack of oxygen (ischemia) resulting from

hypoperfusion. Major ischemic events of therapeutic concern include, but are not limited to, heart attacks and stroke. Ischemia also can affect other tissues, including retinal, liver, kidney, bone, placental and spinal tissue. Prolonged ischemia results in cellular damage, manifest, in the case of cerebral ischemia, as neurological dysfunction. Agents currently used in treatment of stroke are targeted at 1) reversing excessive excitotoxic phenomena associated with an ischemic episode; or 2) increasing blood flow to ischemic tissue.

Ischemic injury may commonly result from *e.g.*, vascular occlusion such as by an embolus or thrombus, hemorrhage, near drowning and near suffocation. Without wishing to be bound by theory, it is believed that ischemia causes a massive release of the excitotoxic amino acid, glutamate, from presynaptic nerve endings in the brain which act on *N*-methyl-D-aspartate (NMDA) receptors on adjacent cells. Once activated, NMDA receptors allow excessive calcium to enter the cell, which in turn activates a number of secondary pathways that ultimately lead to cellular protein degradation and cell death. In the search for effective therapies, efforts have been made to target either the release of presynaptic glutamate (via κ -opiate receptor stimulation), or blockade of NMDA receptor activation (either directly, with NMDA antagonists, or indirectly, with glycine antagonists). Calcium channel blockers and calpain inhibitors have also been investigated. While individual drugs have shown activity in both preclinical and clinical situations, the benefit is limited due to the speed at which the cascade occurs, the time taken for the drugs to be given, and the effectiveness of the therapy. The only drug used clinically to increase blood flow is tissue plasminogen activator (TPA), which aids in the rapid solubilization of clots that are responsible for the vessel blockade. While effective to a limited extent in stroke patients, by actually promoting bleeding, drugs like TPA can be lethal to those patients that have cerebral hemorrhage. As such, TPA cannot be given until the patient has been confirmed as having a stroke rather than hemorrhage. For a stroke patient, the time taken for this analysis to occur obviously increases the length of time of the ischemia and hence the amount of salvageable tissue is reduced. Agents such as NF- κ B activation inhibitors that can act on the ischemic cascade system itself are not limited by such a prolonged diagnosis period, as they are not detrimental in cerebral hemorrhage patients.

Preferred NF- κ B activation inhibitors inhibit NF- κ B activation by the ubiquitin-proteasome pathway. In certain preferred embodiments, the NF- κ B activation

inhibitor inhibits phosphorylation of I κ B- α . In certain preferred embodiments, the NF- κ B activation inhibitor is a proteasome inhibitor. In such embodiments the inhibition of the proteasome is preferably less than complete inhibition. Preferably, the proteasome inhibitor is selected from the group consisting of peptidyl aldehydes, boronic acids, boronic esters, lactacystin, and lactacystin analogs. In certain preferred embodiments, the NF- κ B activation inhibitor is a ubiquitin pathway inhibitor.

The transcription factor NF- κ B is a member of the Rel protein family. The Rel family of transcriptional activator proteins can be divided into two groups. The first group requires proteolytic processing, and includes p105 and p100, which are processed to p50 and p52, respectively. The second group does not require proteolytic processing and includes p65 (Rel A), Rel (c-Rel), and Rel B. NF- κ B comprises two subunits, p50 and an additional member of the Rel gene family, e.g., p 65. Unprocessed p105 can also associate with p65 and other members of the Rel family. In most cells, the p50-p65 heterodimer is present in an inactive form in the cytoplasm, bound to I κ B- α . The ternary complex can be activated by the dissociation and destruction of I κ B- α , while the p65/p105 heterodimer can be activated by processing of p105.

The ubiquitin-proteasome pathway plays an essential role in the regulation of NF- κ B activity, being responsible both for processing of p105 to p50 and for the degradation of the inhibitor protein I κ B- α . (Palombella *et al.*, WO95/25533) In order to be targeted for degradation by the proteasome, I κ B- α must first undergo selective phosphorylation at serine residues 32 and 36, followed by ubiquitination. (Alkalay *et al.*, Proc. Natl. Acad. Sci. USA 92: 10599 (1995); Chen, WO97/35014)

Once activated, NF- κ B translocates to the nucleus, where it plays a central role in the regulation of a remarkably diverse set of genes involved in the immune and inflammatory responses (Grilli *et al.*, International Review of Cytology 143:1-62 (1993)). For example, NF- κ B is required for the expression of a number of genes involved in the inflammatory response, such as TNF- α gene and genes encoding the cell adhesion molecules E-selectin, P-selectin, ICAM, and VCAM (Collins, T., Lab. Invest. (1993) 68:499. NF- κ B is also required for the expression of a large number of cytokine genes such as IL-2, IL-6, granulocyte colony stimulating factor, and IFN- β . Inducible nitric

oxide synthetase is also under regulatory control of NF- κ B.

Proteasome inhibitors block I κ B- α degradation and activation of NF- κ B (Palombella *et al.* WO 95/25533 published 9/28/95; Traenckner, *et al.*, *EMBO J.* (1994) 13:5433). Proteasome inhibitors also block TNF- α induced expression of the leukocyte adhesion molecules E-selectin, VCAM-1, and ICAM-1 (Read, *et al.*, *Immunity* (1995) 2:493). These cell adhesion molecules play a critical role in supporting the emigration of leukocytes from the bloodstream to extravascular sites of injury such as ischemic tissue. Although intended to serve a repair function, the resultant influx of cells, particularly neutrophils, can promote damage by release of cytokines that speed cell death and signal additional cells (e.g., macrophages) to invade the area.

In a second aspect, the present invention is directed to a method of preventing or lessening the severity of reperfusion injury in a mammal comprising administering to said mammal an effective amount of an NF- κ B activation inhibitor. Reperfusion injury may occur as a result of one or more of the following events: cellular acidosis leading to calcium overload; increased intracellular osmotic loads of catabolites leading to cell swelling; free radicals from neutrophils and other inflammatory cells. Preferred NF- κ B activation inhibitors are selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, inhibitors of serine phosphorylation of I κ B- α . and mixtures thereof.

In a third aspect, the present invention is directed to a method of preventing, reducing the size of, or lessening the severity of infarction in a mammal comprising administering to said mammal an effective amount of an NF- κ B activation inhibitor. Preferred NF- κ B activation inhibitors are selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, inhibitors of serine phosphorylation of I κ B- α . and mixtures thereof. In preferred embodiments, the method according to this aspect of the invention prevents, reduces the size or lessens the severity of infarction after occlusion of a cerebral vessel or a cardiac vessel. In certain preferred embodiments, the method prevents

the occlusion from resulting in stroke, or lessens the severity of a stroke resulting from cerebral vessel occlusion.

The most common form of stroke is thrombotic stroke, where occlusion of cerebral blood vessels is believed to be caused by a plug of aggregated platelets. Often, these platelet plugs are released as emboli from platelet thrombi on atherosclerotic plaques in major carotid or cerebral vessels. Thrombotic strokes often have a characteristic "stuttering" onset in which an initial modest, often reversible, neurological deficit is followed by a more severe, irreversible stroke. The initial event often reflects transient cerebrovascular obstruction by platelet thrombi, which is potentially reversible. Indeed, clinically, a mild stroke can be viewed as the extreme end of the spectrum of transient ischemic attacks (TIA) - a reversible neurological deficit in which a cerebral vessel is transiently occluded by an embolic platelet thrombus, which subsequently disaggregates, thus allowing flow to be reestablished. Therefore, the administration of an agent as disclosed herein after the onset of transient vascular occlusion is contemplated by the present invention. Another important form of stroke is stroke after cerebral hemorrhage, as discussed above. It is believed that the agents disclosed herein will have broad range efficacy in preventing, reducing the size, or lessening the severity of infarcts resulting from a variety of causes, including thrombotic stroke and stroke following cerebral hemorrhage. As a practical matter, the reduction of infarct size or lessening of infarct severity will be inferred from a reduction in symptoms associated with the infarct, including without limitation neurological symptoms and cardiac performance symptoms.

In a fourth aspect, the present invention is directed to a method to treating ischemia or reperfusion injury, including without limitation reducing the size or lessening the severity of infarction in a mammal comprising administering to the mammal an adjunct therapeutic, in addition to administering an NF- κ B activation inhibitor. Preferred NF- κ B activation inhibitors are selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, inhibitors of serine phosphorylation of I κ B- α . and mixtures thereof. Certain preferred adjunct therapeutics include without limitation, agents which such as steroids which further inhibit NF- κ B activation or inhibit the expression or action of proinflammatory cytokines or cellular adhesion molecules; agents which act to either reperfuse or oxygenate tissues, antiedema drugs, thrombolytics such as TPA, streptokinase and urokinase, polyanions such as heparin, anticoagulants; and agents that assist in temperature normalization. Agents that inhibit the action of cytokines or cellular adhesion

molecules include, without limitation, antibodies, or an antibody derivative, which may more preferably be a monoclonal antibody, a human antibody, a humanized antibody, a single-chain antibody, a chimeric antibody, or an antigen-binding antibody fragment. The use of any of the agents discussed or
5 disclosed herein in combination with any other agent or agents used in the treatment of stroke or myocardial infarction is further contemplated within the scope of the present invention.

In the present description, the following definitions will be used.

10 "Treatment" shall mean preventing or lessening ischemic injury or reperfusion injury, including the prevention of infarction or reduction in size or lessening in severity of infarct, including without limitation infarct after vascular occlusion. Any amelioration of any symptom of the infarct pursuant to treatment using any proteasome inhibitor, ubiquitin pathway inhibitor, or agent that interferes with activation of NF- κ B via the ubiquitin
15 proteasome pathway is within the scope of the invention.

The term "mammals" is intended to include humans.

20 "Inhibitors of NF- κ B activation" or "NF- κ B activation inhibitors" shall mean any substance which inhibits of NF- κ B activation via the ubiquitin proteasome pathway, and shall include any substance that 1) inhibits the proteasome or the activity thereof; 2) inhibits ubiquitination of I κ B- α or p105; or 3) inhibits phosphorylation of I κ B- α or p105.

25 "Ubiquitin pathway inhibitor" shall mean any substance which directly or indirectly inhibits ubiquitination or the transfer of ubiquitin to proteins. Non-limiting examples of ubiquitin pathway inhibitors include those disclosed in Berleth et al, *Biochem.* 35(5):1664-1671, (1996). Inhibitors of I κ B- α phosphorylation are also known (Chen,
Cell 84:853 (1996)).

30 "Proteasome inhibitor" shall mean any substance which directly or indirectly inhibits the proteasome or the activity thereof. Non-limiting examples of proteasome inhibitors for use in the present invention include peptide aldehydes (Stein et al. WO 95/24914 published September 21, 1995; Siman et al. WO 91/13904 published September 19, 1991; Iqbal et al. *J. Med. Chem.* 38:2276-2277 (1995)), peptide boronic acids (Adams

et al. WO 96/13266 published May 9, 1996; Siman et al. WO 91/13904), lactacystin, and lactacystin analogs (Fenteany et al. Proc. Natl. Acad. Sci. USA (1994) 91:3358; Fenteany et al. WO 96/32105, published 10/17/96).

5 Peptide aldehyde proteasome inhibitors for use in the present invention preferably are those disclosed in Stein et al. WO 95/24914 published September 21, 1995 or Siman et al. WO 91/13904 published September 19, 1991, both hereby incorporated by reference in their entirety.

10 Boronic acid or ester compounds for use in the present invention preferably are those disclosed in Adams et al. WO 96/13266 published May 9, 1996, or Siman et al. WO 91/13904, both of which are hereby incorporated by reference in their entirety.

15 More preferably, the boronic acid compound for use in the present invention is selected from the group consisting of :

N-(4-morpholine)carbonyl- β -(1-naphthyl)-L-alanine-L-leucine boronic acid

15 N-(8-quinoline)sulfonyl- β -(1-naphthyl)-L-alanine -L-alanine-L-leucine boronic acid,

N-(2-pyrazine)carbonyl-L-phenylalanine-L-leucine boronic acid, and

N-(4-morpholine)carbonyl-[O-(2-pyridylmethyl)]-L-tyrosine-L-leucine boronic acid.

20 Lactacystin and lactacystin analog compounds for use in the present invention preferably are those disclosed in Fenteany et al. WO 96/32105, published 10/17/96, hereby incorporated by reference in its entirety. More preferably, the lactacystin analog is selected from lactacystin, clasto-lactacystin β -lactone, 7-ethyl-clasto-lactacystin β -lactone and 7-n-propyl-clasto-lactacystin β -lactone are used for the methods of the invention. Most preferably the lactacystin analog is 7-n-propyl-clasto-lactacystin β -lactone.

25 The agents disclosed herein may be administered by any route, including intradermally, subcutaneously, orally, intraarterially or intravenously. Preferably, administration will be by the intravenous route. Preferably parenteral administration may be provided in a bolus or by infusion.

30 The concentration of a disclosed compound in a pharmaceutically acceptable mixture will vary depending on several factors, including the dosage of the compound to be administered, the pharmacokinetic characteristics of the compound(s) employed, and the route of administration. Effective amounts of agents for treating ischemia or reperfusion injury would broadly range between about 10 μ g and about 50 mg per Kg of body weight of a recipient mammal. The agent may be administered in a single dose or in repeat doses. Treatments may be administered daily or more frequently depending upon a number of

factors, including the overall health of a patient, and the formulation and route of administration of the selected compound(s).

The disclosed compound may be administered at any time before, during, or after the onset of ischemia. In certain preferred embodiments, the agent is administered after the onset of ischemia, but at a time early enough to reverse damage to some or all of the affected tissue. Preferably, the agent is administered less than 12 hours, more preferably less than 6 hours and still more preferably less than about 3 hours after onset of the ischemic event. Treatment may be initiated before, during or after reperfusion of the ischemic tissue. In many instances, the time of reperfusion cannot be accurately determined, but it is preferred that treatment begin before, during or soon after reperfusion to prevent or lessen additional damaging consequences which may result from reperfusion.

In the event of a clot inducing an ischemic episode, drugs such as TPA which break up the clot can be administered to reduce the potential tissue damage. Once dosed, the drug acts quickly to remove the vascular blockade and, therefore, the time at which the ischemic event ends can be determined. In one preferred embodiment, the inhibitor of NF- κ B activation is administered at the same time or immediately following the clot dissolving drug.

In certain other preferred embodiments, the inhibitor of NF- κ B activation is administered prior to the onset of ischemia. The onset of ischemia can be predicted in the case of certain medical procedures, such as surgical procedures. In another preferred embodiment, the disclosed compound is administered just prior to or immediately following the release of ischemia and onset of reperfusion during such a medical procedure (e.g., angioplasty procedures).

The following examples are intended to further illustrate certain preferred embodiments of the invention and are not limiting in nature.

EXAMPLES

Example 1

5 Six male Sprague Dawley rats (300 g) were anesthetized with haloethane and subjected to middle cerebral artery (MCA) occlusion using a nylon filament for 2 h. Subsequently, the filament was removed and reperfusion of the infarcted tissue occurred for 24 hours before the rat was sacrificed.

Staining of coronal sections (2.0 mm × 7-8) with triphenyltetrazolium chloride (TTC) taken throughout the brain were evaluated under blinded conditions using image analysis to determine infarct size.

10 The infarct was also expressed as a percentage of the contralateral (non-infarcted) hemisphere to provide an indication of how much of the ipsilateral (infarcted) hemisphere was actually damaged by the procedure. Because edema is present in the infarcted hemisphere, it is often impossible to directly ascertain the percentage of the ipsilateral hemisphere that has been damaged.

Dosing Regimen

15 Rats were given *iv* bolus injections (1.0 mL/kg) of either vehicle (10% PEG 200/saline; n = 3) or *N*-(2-pyrazine)carbonyl-L-phenylalanine-L-leucine boronic acid (0.03 mg/kg; n = 3) at 30 minutes, 2 hours, and 6 hours after the start of the occlusion.

Results

20 Infarct volume was decreased by 62% on the ipsilateral hemisphere in treated animals (Figure 1). This reflects a decrease in total damage of the hemisphere from 19% to 2% (Figure 2).

Example 2

25 *Methods*

Male Sprague Dawley rats (250-400 g) were anesthetized with haloethane and subjected to middle cerebral artery (MCA) occlusion using a nylon filament for 2 h. Subsequently, the filament was removed and reperfusion of the infarcted tissue occurred for 24 hours before the rat was sacrificed.

30 Immediately after the filament was withdrawn, the animals were evaluated using a neurological scoring system. Neurological scores were expressed on a scale from 0 to 10, with 0 representing no neurological deficit and 10 representing severe neurological deficit. After 24 hours and before sacrifice, animals were evaluated a second time using the same neurological scoring system.

Staining of coronal sections (2.0 mm × 7-8) with triphenyltetrazolium chloride (TTC) taken throughout the brain were evaluated under blinded conditions using image analysis to determine infarct size.

5 *Dosing Regimen*

Rats were given *iv* bolus injections (1.0 mL/kg) of either vehicle (50% propylene glycol/saline; n = 8) or 7-n-propyl-*clasto*-lactacystin β-lactone (0.1 mg/kg; n = 6) at 2 hours after the start of the occlusion.

10 *Results*

In animals treated with 7-n-propyl-*clasto*-lactacystin β-lactone, infarct volume was decreased by 70% (Figure 5).

All animals had a neurological score of 10 ± 0 immediately after the 2 hour ischemic episode. At 24 hours, the vehicle-treated rats had a mean score of 8.7 ± 0.6 , whereas rats 15 treated with a single 0.1 mg/kg dose of 7-n-propyl-*clasto*-lactacystin β-lactone had a mean score of 5.5 ± 1 (Figure 6). These data represent a 40% neurological improvement for the drug-treated animals.

Conclusion

20 7-n-propyl-*clasto*-lactacystin β-lactone provides significant protection in both the degree of neurological deficit and infarcted brain damage.

Example 3

25 *Methods*

Male Sprague Dawley rats (250-400 g) were anesthetized with haloethane and subjected to middle cerebral artery (MCA) occlusion using a nylon filament for 2 h. Subsequently, the filament was removed and reperfusion of the infarcted tissue occurred for 24 hours before the rat was sacrificed.

30 Immediately after the filament was withdrawn, the animals were evaluated using a

neurological scoring system. Neurological scores were expressed on a scale from 0 to 10, with 0 representing no neurological deficit and 10 representing severe neurological deficit. After 24 hours and before sacrifice, animals were evaluated a second time using the same neurological scoring system.

5 Staining of coronal sections (2.0 mm × 7-8) with triphenyltetrazolium chloride (TTC) taken throughout the brain were evaluated under blinded conditions using image analysis to determine infarct size.

Dosing Regimen

10 Rats were given *iv* bolus injections (1.0 mL/kg) of either vehicle (50% propylene glycol/saline; n = 8) or 7-n-propyl-*clasto*-lactacystin β -lactone (0.3 mg/kg; n = 7) at 2 hours after the start of the occlusion. Two additional groups of rats were given *iv* bolus injections (1.0 mL/kg) of 7-n-propyl-*clasto*-lactacystin β -lactone at 0 minutes, 2 hours, and 6 hours after the start of the occlusion. One group (0.1 mg/kg × 3; n = 6) received 0.1
15 mg/kg at each of these times, while another group (0.3 mg/kg × 3; n = 7) received 0.3 mg/kg at each of the three timepoints.

Results

20 In animals treated with a single dose of 7-n-propyl-*clasto*-lactacystin β -lactone, infarct volume was decreased by 50% (Figure 3). Infarct volume was not significantly decreased in either the 0.1 mg/kg × 3 dosage group or the 0.3 mg/kg × 3 dosage group (Figure 7).

25 All animals had a neurological score of 10 ± 0 immediately after the 2 hour ischemic episode. At 24 hours, the vehicle-treated rats had a mean score of 8.7 ± 0.6, whereas rats treated with a single 0.3 mg/kg dose of 7-n-propyl-*clasto*-lactacystin β -lactone had a mean score of 4 ± 1 (Figure 8). These data represent a 60% neurological improvement for the drug-treated animals. No significant improvement in neurological score was observed in either the 0.1 mg/kg × 3 dosage group or the 0.3 mg/kg × 3 dosage group (Figure 8).

Conclusion

30 7-n-propyl-*clasto*-lactacystin β -lactone provides significant protection in both the degree of neurological deficit and infarcted brain damage.

Although the foregoing refers to particular preferred embodiments, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that various modifications may be made to the disclosed embodiments and that such modifications are intended to be within the scope of the present invention, which is defined by the following claims.

CLAIMS :

1. A method of treating ischemic injury in a mammal comprising administering to said mammal an effective amount of an NF- κ B activation inhibitor selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF- κ B via the ubiquitin proteasome pathway, and mixtures thereof.
2. A method of treating reperfusion injury after ischemia in a mammal comprising administering to said mammal an effective amount of an NF- κ B activation inhibitor selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF- κ B via the ubiquitin proteasome pathway, and mixtures thereof.
3. A method of preventing, reducing the size or lessening the severity of infarct resulting from ischemia or reperfusion injury in a mammal comprising administering to said mammal an effective amount of an NF- κ B activation inhibitor selected from the group consisting of proteasome inhibitors, ubiquitin pathway inhibitors, agents that interfere with the activation of NF- κ B via the ubiquitin proteasome pathway, and mixtures thereof.
4. The method of claims 1-3 wherein the ischemia is the result of vascular occlusion.
5. The method of claim 4 wherein the occlusion is of a cerebral vessel.
6. The method of claim 4 wherein the occlusion is of a cardiac vessel.
7. The method of claim 1-3 wherein the method prevents or lessens the severity of stroke.
8. The method of claim 1-3 wherein the method prevents or lessens the severity of stroke resulting from the occlusion of the cerebral vessel.
9. The method of claim 6 wherein the method prevents or lessens the severity of myocardial infarction resulting from the occlusion of a cardiac vessel.

10. The method of claim 1-3 wherein the NF- κ B activation inhibitor is an agent that inhibits phosphorylation of I κ B- α .

5 11. The method of claims 1 - 3 wherein the NF- κ B activation inhibitor comprises a proteasome inhibitor.

12. The method of claim 11 wherein said proteasome inhibitor is a peptidyl aldehyde.

10 13. The method of claim 11 wherein said proteasome inhibitor is a peptidyl boronic acid or peptidyl boronic ester.

14. The method of claim 11 wherein said proteasome inhibitor is a lactacystin analog.

15 15. The method of claim 13 wherein said proteasome inhibitor is N-(2-pyrazine) carbonyl-L-phenylalanine-L-leucine boronic acid.

16. The method of claim 14 wherein the proteasome inhibitor is 7-n-propyl-*clasto*-lactacystin β -lactone.

20 17. The method of claims 1-3 wherein the NF- κ B activation inhibitor is administered to the mammal less than 12 hours after the onset of ischemia.

25 18. The method of claims 1-3 wherein the NF- κ B activation inhibitor is administered to the mammal less than 6 hours after the onset of ischemia.

19. The method of claims 1-3 wherein the NF- κ B activation inhibitor is administered to the mammal before the onset of ischemia.

30 20. The method of claims 1-4 further comprising the step of administering a second

21. The method of claim 21 wherein the second agent is selected from the group consisting of NF- κ B activation inhibitors, agents which inhibit the expression or action of proinflammatory cytokines or cellular adhesion molecules, agents which act to reperfuse or oxygenate tissues, and agents which assist in temperature normalization.

5 22. The method of claim 21 wherein the second agent is selected from the group consisting of steroids, antiedema drugs, thrombolytics, clot solubilizing drug, polyanions and anticoagulants.

10 23. The method of claim 22 wherein the second agent comprises a thrombolytic or clot solubilizing drug.

15 24. The method of claim 24 wherein the thrombolytic clot solubilizing drug comprises tissue plasminogen activator.

FIGURE 1

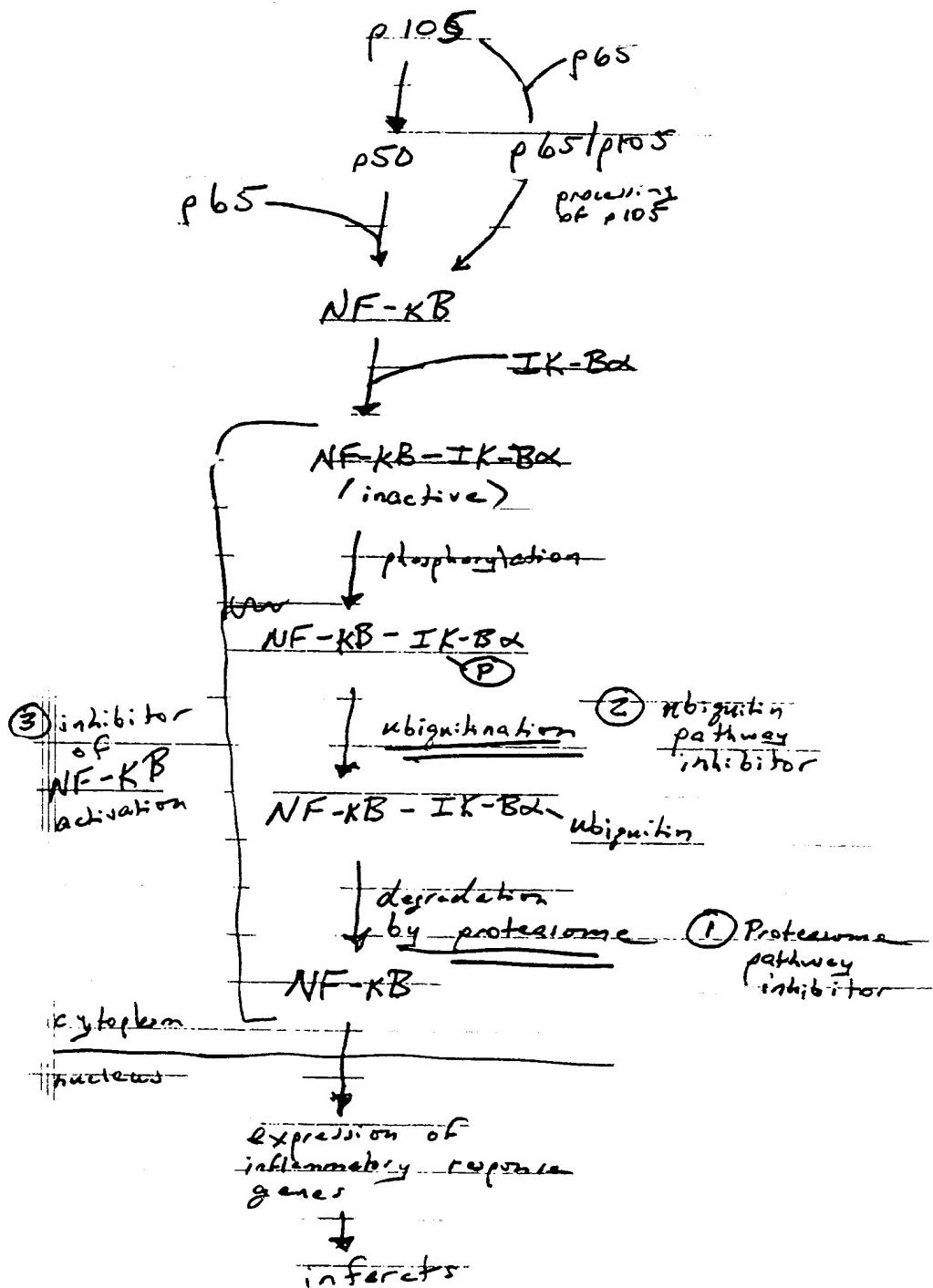
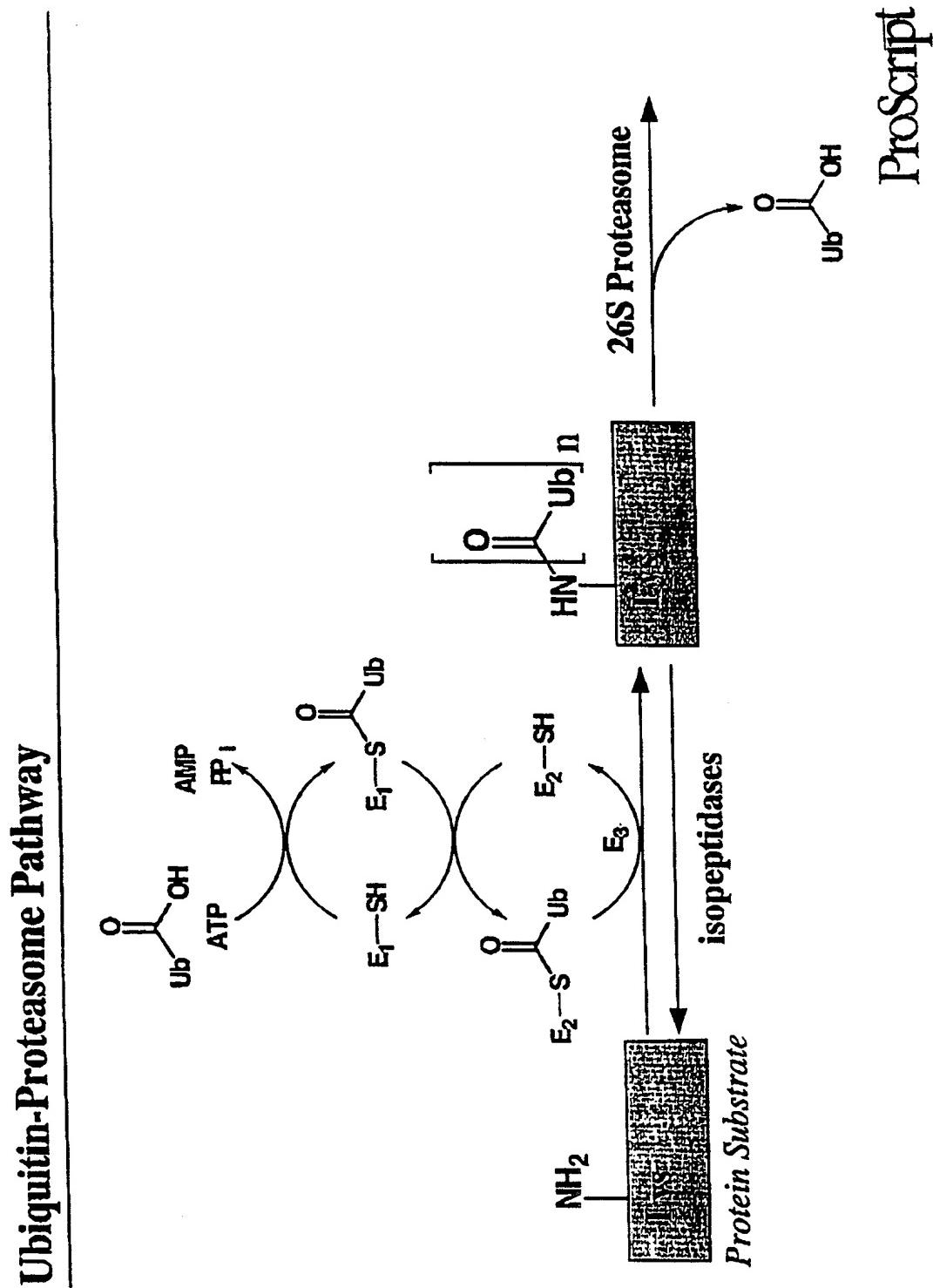


FIGURE 2



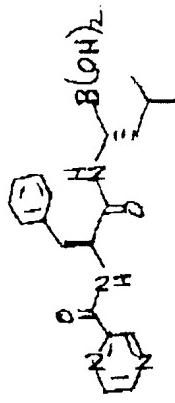
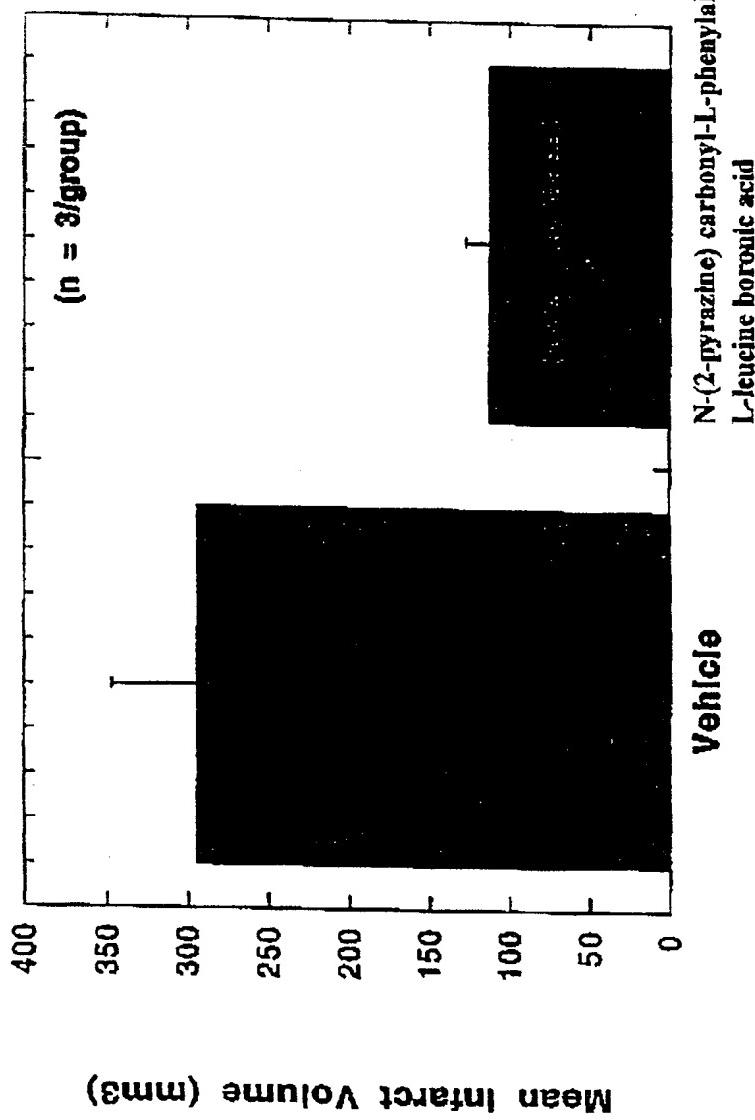


Figure 3.

Effect of N-(2-pyrazine) carbonyl-L-phenylalanine-L-leucine boronic acid in a Rat Stroke Model (0.03 mg/kg, i.v. @ 0.5, 2 & 6 after MCA occlusion)



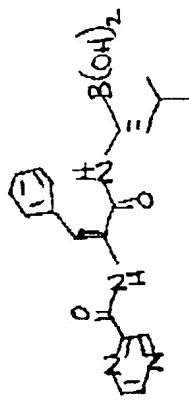
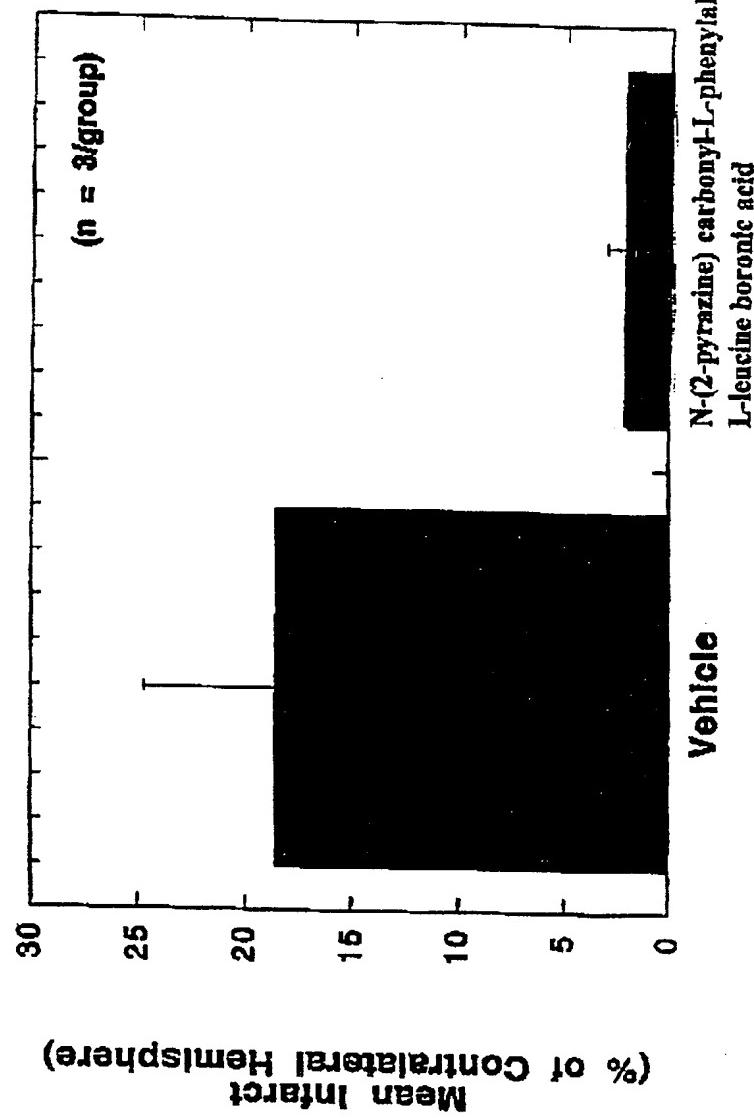


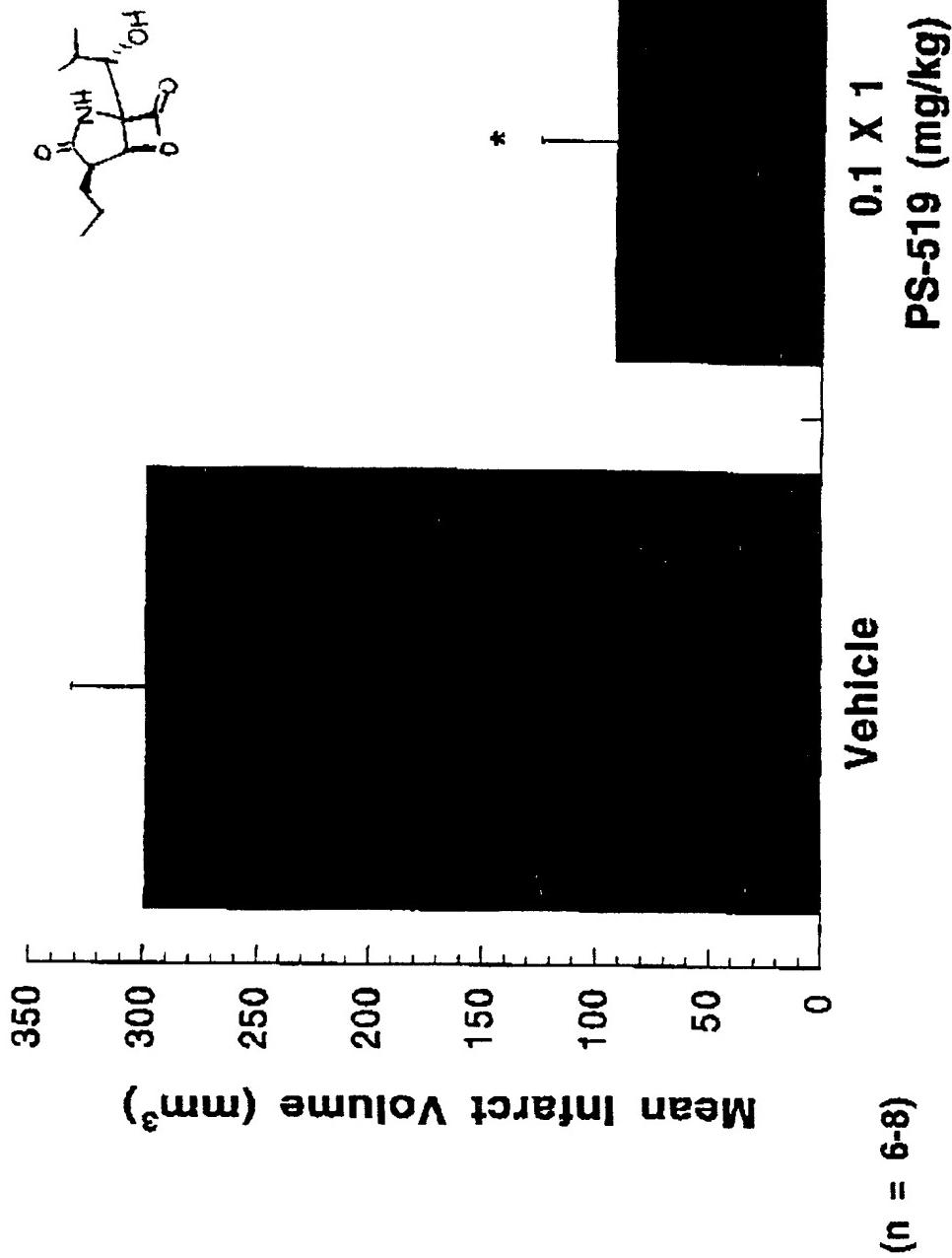
Figure 4.

Effect of N-(2-pyrazine) carbonyl-L-phenylalanine-L-leucine boronic acid in a Rat Stroke Model (0.03 mg/kg, i.v. @ 0.5, 2 & 6 after MCA occlusion)



7-n-propyl-clasto-lactacystin β -lactone

Effect of A (IV) on Infarct Volume in Rats



7-n-propyl- α -lactoyl- β -lactone

Effect of A (IV) on Neurological Score

Figure 6

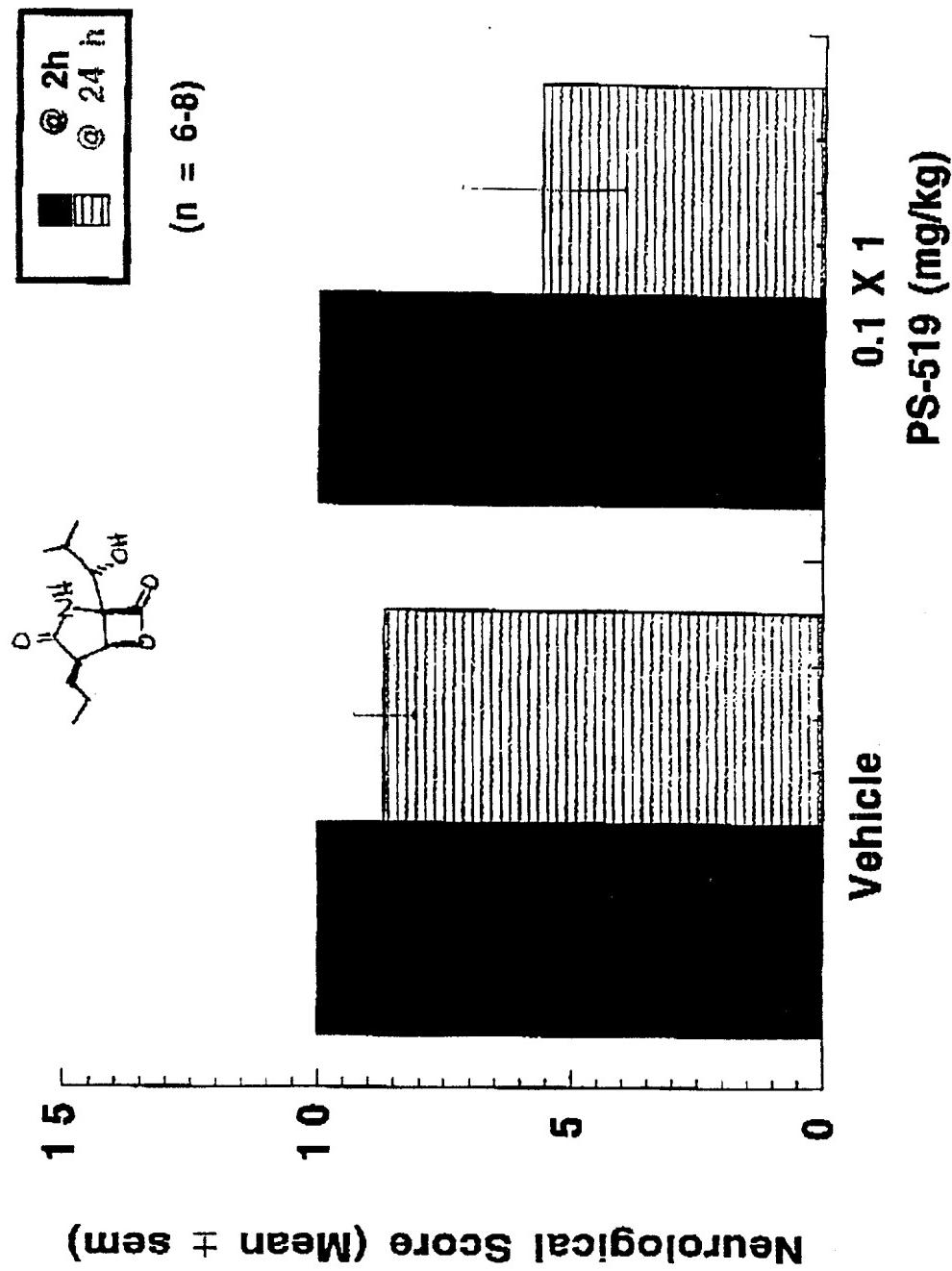
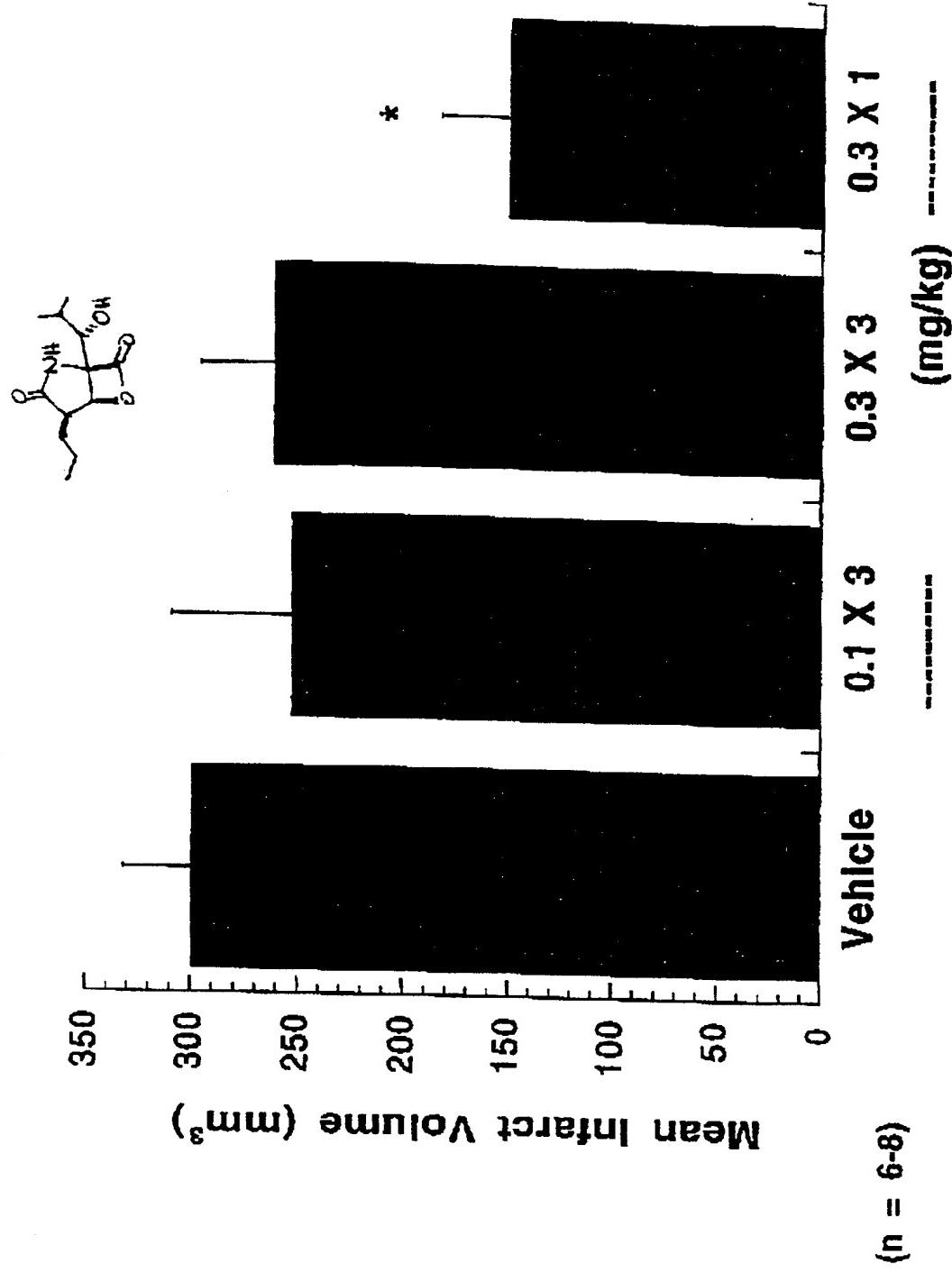


Figure 7.

7-n-propyl-delta-lactacytin β -lactone

Effect of A (IV) on Infarct Volume in Rats



7-n-propyl-clathro-lactacyclin β -lactone

Effect of A (IV) on Neurological Score

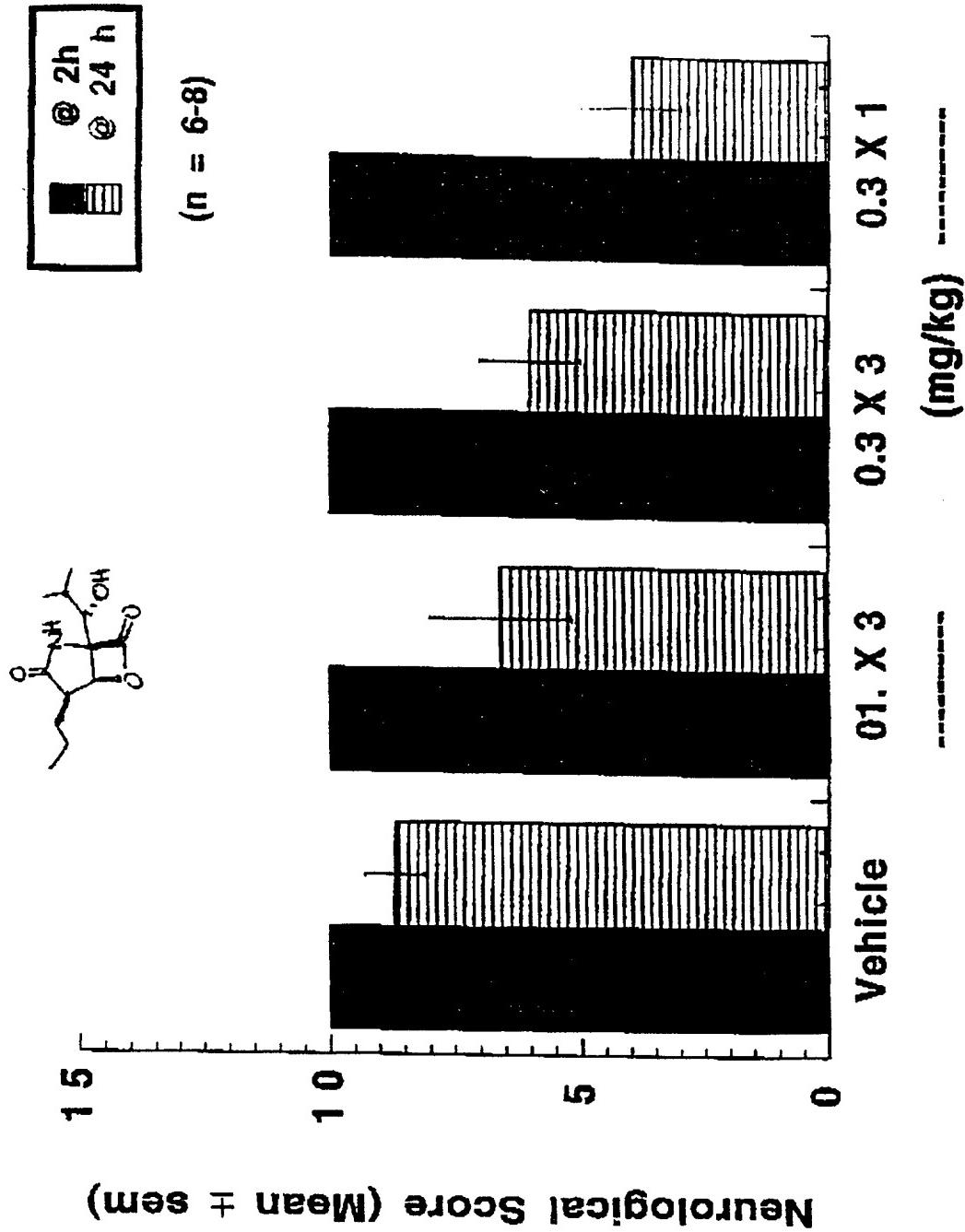


Figure 9.

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/US 98/03077

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K38/05 A61K31/40

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 35430 A (FUJISAWA PHARMACEUTICAL CO ;MORISHITA RYUICHI (JP); OGAWARA TOSHIRO) 14 November 1996 & EP 0 824 918 A ---	
A	WO 96 13266 A (PROSCRIPT INC) 9 May 1996 cited in the application see page 4, line 9 - page 6, line 4 ---	
A	WO 96 32105 A (HARVARD COLLEGE ;SCHREIBER STUART L (US); STANDAERT ROBERT F (US);) 17 October 1996 cited in the application see page 2, line 2 - line 6 see page 81, line 2 - page 91, line 14 --- -/-	

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search

29 May 1998

Date of mailing of the international search report

24.06.98

Name and mailing address of the ISA

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Authorized officer

Sitch, W

INTERNATIONAL SEARCH REPORT

Intern: al Application No

PCT/US 98/03077

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 95 25533 A (HARVARD COLLEGE) 28 September 1995 cited in the application see page 8, line 23 - page 9, line 19 ---	
A	ALKALAY ET AL: "STIMULATION -DEPENDENT IKAPPABALPHA PHOSPHORYLATION MARKS THE NF-KAPPAB INHIBITOR FOR DEGRADATION VIA THE UBIQUITIN-PROTEASOME PATHWAY" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, vol. 92, 1995, pages 10559-10603, XP002066578 cited in the application see page 10599 see abstract ---	
A	CHEN ET AL: "SITE-SPECIFIC PHOSPHORYLATION OF IKAPPABALPHA BY A NOVEL UBIQUITINATION-DEPENDENT PROTEIN KINASE ACTIVITY" CELL, vol. 84, 22 March 1996, pages 853-862, XP002066579 cited in the application see page 853 see abstract ---	
A	BERLETH ET AL: "MECHANISM OF UBIQUITIN CONJUGATING ENZYME E2-230K: CATALYSIS INVOLVING A THIOL RELAY?" BIOCHEMISTRY, vol. 35, 1996, pages 1664-1671, XP002066580 cited in the application see page 1664 see abstract ---	
A	KOONG ET AL: "HYPOXIA CAUSES THE ACTIVATION OF NUCLEAR FACTOR KAPPAB THROUGH THE PHOSPHORYLATION OF IKAPPABALPHA ON TYROSINE RESIDUES" CANCER RESEARCH, vol. 54, 1994, pages 1425-1430, XP002066581 see page 1425 see abstract -----	

INTERNATIONAL SEARCH REPORT

Int'l application No.
PCT/US 98/03077

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

- 1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
Although claims 1-24 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
- 2. Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
- 3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This international Searching Authority found multiple inventions in this international application, as follows:

- 1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
- 2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
- 3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
- 4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- The additional search fees were accompanied by the applicant's protest.
- No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat	al Application No
PCT/US 98/03077	

Patent document cited in search report	Publication date	Patent family member(s)			Publication date
WO 9635430	A 14-11-1996	EP	0824918 A		25-02-1998
WO 9613266	A 09-05-1996	AU CA EP FI NO ZA	4139896 A 2203936 A 0788360 A 971746 A 971929 A 9509119 A		23-05-1996 09-05-1996 13-08-1997 06-06-1997 12-06-1997 27-05-1996
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WO 9525533	A 28-09-1995	AU AU CA EP JP	682264 B 2121595 A 2184727 A 0750507 A 9510710 T		25-09-1997 09-10-1995 28-09-1995 02-01-1997 28-10-1997